

Characterization of Two Genetically Separable Inorganic Phosphate Transport Systems in *Escherichia coli*

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Inorganic phosphate (P_i) transport by wild-type cells of *Escherichia coli* grown in excess phosphate-containing media involves two genetically separable transport systems. Cells dependent upon the high affinity-low velocity Pst (phosphate specific transport) system have a K_m of $0.43 \pm 0.2 \mu M P_i$ and a V_{max} of 15.9 ± 0.3 nmol of P_i (mg [dry weight] $^{-1}$ min $^{-1}$) and will grow in the presence of arsenate in the medium. However, cells dependent upon the low affinity-high velocity Pit (P_i transport) system have a K_m of $38.2 \pm 0.4 \mu M$ and a V_{max} of 55 ± 1.9 nmol of P_i (mg [dry weight] $^{-1}$ min $^{-1}$), and these cells cannot grow in the presence of an arsenate-to- P_i ratio of 10 in the medium. P_i transport by both systems was sensitive to the energy uncoupler 2,4-dinitrophenol and the sulfhydryl reagent *N*-ethylmaleimide, whereas only the Pst system was very sensitive to sodium cyanide. Evidence is presented that P_i is transported as P_i or a very labile intermediate and that accumulated P_i does not exit through the Pst or Pit systems from glucose-grown cells. Kinetic analysis of P_i transport in the wild-type strain containing both the Pst and Pit transport systems revealed that each system was not operating at full capacity. In addition, P_i transport in the wild-type strain was completely sensitive to sodium cyanide (a characteristic of the Pst system).

P_i plays a key role in the metabolism of all cells. Thus it is not surprising that many cells have evolved multiple systems for P_i transport and accumulation. To determine how these systems function and interact in vivo, we have characterized P_i transport in *Escherichia coli* grown under conditions of balanced growth (8).

In this study we concentrated on two major P_i transport systems in *E. coli* which can carry out active transport of P_i against a 1,000-fold concentration gradient. These systems did not seem to be induced by any carbon source; only the concentration of P_i in the growth medium affected their expression. The two major P_i transport systems were genetically separable: the *pst* (phosphate specific transport) gene is located at min 83 and the *pit* (P_i transport) genes are located at min 76 on the *E. coli* genetic map (1).

A mutation which completely inactivates the Pit system has been found in *E. coli* strain K-10 (20). In addition, we have isolated arsenate-resistant strains which contain *pit* mutations (2). Sprague et al. (18) isolated three mutants which do not have an active Pit system after selecting for strains with altered fatty acid metabolism. Although the three mutants have similar phenotypes and the mutations map in the same area

of the *E. coli* chromosome (min 76), it has not been determined at this time whether all three mutations occur in the same gene.

The Pst system for P_i transport involves at least three genes. Two of these genes, *phoS* and *phoT*, are involved also in the regulation of the synthesis of alkaline phosphatase, a periplasmic protein produced in greatest quantity under conditions of P_i starvation. *phoS* codes for the phosphate-binding protein, which functions as part of the Pst system. Alterations in the *phoS* protein cause alkaline phosphatase to be produced constitutively. Two types of mutations which inactivate the Pst system have been studied in this laboratory. One class, *pst*, was isolated in arsenate-resistant mutants (2), whereas the second class, *phoT*, was isolated as alkaline phosphatase constitutive mutants (20). These two classes of mutations produce different levels of alkaline phosphatase constitutively. Two types of *phoT* mutants having different phenotypes with respect to P_i transport have also been studied by A. M. Torriani (submitted for publication), but complementation tests among these four alleles have not yet been performed.

A detailed kinetic analysis of P_i transport through the Pst and Pit systems in cells grown in glucose minimal salts medium with 1 mM phosphate as the sole source of phosphate is presented. In addition we have studied the re-

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sponse of the P_i transport systems to various inhibitors.

MATERIALS AND METHODS

Strains and media. The strains used in this study are listed in Table 1. ML broth (20) was the rich medium. WT, a minimal salts medium (2) (pH 7.5), containing no phosphate, supplemented with 0.6% glucose, 1 mM P_i, 2 µg of thiamine per ml, and 50 µg each of proline, leucine, adenine, and tryptophan per ml, was the excess phosphate medium used, unless otherwise stated in the figure legends.

Mating procedure. Cells for conjugation were grown in ML broth at 37°C. The Hfr donor strain was grown with slow agitation whereas the F⁻ strain was grown with vigorous aeration. When the optical density at 600 nm (OD₆₀₀) of each culture had reached 0.6 to 0.8, 3 ml of each strain was mixed in a 500-ml flask and incubated at 37°C for 60 to 90 min without shaking. Recombinants were obtained by plating dilutions of the mating mixture on the appropriate selection media. Recombinant colonies were purified by restreaking on the selective medium and then tested for nonselected markers by replica plating.

Determination of dry weight. The value used in dry weight determination was obtained from Stock et al. (19), who worked at 500 nm. This value was converted to 0.32 mg (dry weight) per ml at OD₆₀₀ = 1 by comparing a bacterial culture growing in minimal medium at both wavelengths.

Chemicals. Salicin and streptomycin sulfate were purchased from Schwartz/Mann. DL-α-Glycerophosphate was purchased from Sigma Chemical Co., and Tris was from Research Plus Labs. Carrier-free ³²P_{O₄³⁻ in dilute HCl was purchased from New England Nuclear Corp., and ⁷⁴AsO₄³⁻ was obtained in isocitrate buffer from Amersham Corp.}

Chemical assays: P_i. The method of Berenblum and Chain (3) was employed in the chemical assay of P_i. The sample, in 3 ml, was mixed with 1 ml of 0.1 M NaCl, 0.5 ml of 1 N H₂SO₄, 0.5 ml of 5% (wt/vol) (NH₄)₆-Mo₇O₂₄·4H₂O, and 3 ml of isobutanol, with thorough mixing after each addition. A 1-ml volume of the organic phase was then added to 0.5 ml of SnCl₂ solution (which was prepared fresh daily by a 1:200 dilution into 1 N H₂SO₄ of a stock solution of 10 g of SnCl₂ in 25 ml of concentrated HCl). The sample was clarified by brief centrifugation. The absorbance of the organic phase at 725 nm was measured and compared to a standard phosphate curve prepared for each assay set (0 to 60 µM P_i).

Transport assays. Single colonies picked from ML agar plates were grown to log phase (OD₆₀₀ of 0.4 to 0.8) in ML broth and then used to inoculate the desired minimal medium supplemented with 0.1% Difco Casamino Acids. Cultures were allowed to grow to stationary phase at 37°C and then used to inoculate the same minimal medium without the Casamino Acids. To avoid a long lag phase, cells were grown at 37°C for 10 to 16 h, collected by centrifugation, and suspended in a small volume of WT medium before inoculation into fresh WT medium with a 1% final concentration of the desired carbon source (usually glucose). Deviations from this procedure are described in the figure legends. Cultures were grown with aeration at 37°C, and growth was monitored spectrophotometrically.

Phosphate accumulation. A working solution of ³²P_i, diluted with nonradioactive phosphate (specific activity = 10³ to 10⁴ cpm/nmol), was prepared at least 24 h before each experiment. This solution was filtered three times through membrane filters (Millipore Corp., type HA, 0.45 µm pore size) just before use (15). For transport assays cells were grown to mid-log phase. A constant cell density was maintained by dilution with prewarmed medium (37°C), if necessary. A 10-ml sample of cells was filtered on a membrane filter (Millipore Corp., 0.45 µm pore size), washed with 10 ml of prewarmed WT medium, and suspended in WT medium containing 100 µg of chloramphenicol per ml and 0.6% glucose. This suspension was incubated for 3 min at 37°C with aeration before ³²P_i was added. At suitable intervals samples were collected on membrane filters and washed with WT medium containing 1 mM P_i. The filters were secured to planchets by dissolving them with 1 ml of acetone which was evaporated by placing the planchets under a heat lamp. Radioactivity was determined in a Nuclear Chicago Gas-Flow counter (model 1050). For K_m and K_i determinations, the initial velocity was calculated from three or four experimental points taken between 0.2 and 1.2 min.

Chromatography. Descending chromatography was carried out in 0.03 M ammonium acetate in 95% ethanol. Whole cell samples were collected on nitrocellulose filters, washed, and placed on dry ice to stop all metabolic activity. A piece of Whatman 3MM filter paper was cut into two portions, and the filter was sewn to each part so that the solvent would be forced to run through the membrane filter. This paper was left on a descending chromatography tank for 15 to 25 h until the solvent front had moved at least 30 cm.

TABLE 1. *Strains used*

Strain	Relevant genotype ^a	Source or reference
GR2131B	F ⁻ <i>glpT</i> <i>pst</i> ⁺ <i>pit</i> ⁺ <i>mtl</i> <i>rpsL</i> <i>bglR</i>	Spontaneous Bgl ⁺ from GR2131 (19)
GR5172	F ⁻ <i>glpT</i> <i>pst</i> ⁺ <i>pit</i> <i>mtl</i> ⁺ <i>rpsL</i> <i>bglR</i>	This study
GR5178	F ⁻ <i>glpT</i> <i>pst</i> <i>pit</i> ⁺ <i>mtl</i> ⁺ <i>rpsL</i> <i>bglR</i> ⁺	This study
UR1	HfrC <i>glpT</i> <i>pst</i> <i>pit</i> <i>mtl</i> ⁺ <i>rpsL</i> ⁺ <i>bglR</i> ⁺	Bennett and Malamy(2)

^a The conventions of Demerec et al. (4) have been used to describe all cell genotypes. Note that *bglR* represents the mutant genotype which confers the ability to use β-glucosides (salicin), and *bglR*⁺ represents the wild-type genotype (unable to utilize salicin). The nomenclature in the latest *E. coli* linkage map (1) has also been followed. Only the portions of the genotype relevant to this study are listed for each strain. For additional markers see reference 20.

The chromatogram was then dried in an oven and placed on X-ray film.

Electrophoresis. High-voltage electrophoresis was carried out at 2 kV for 1 to 2 h in a cooled Savant flat plate paper electrophoresis apparatus using the procedure of Rae and Strickland (12). The paper was saturated with the same buffer (0.35 ml of 98% formic acid and 43.5 ml of glacial acetic acid made to 1 liter with water) used to fill the electrode chambers. Samples for electrophoresis were obtained by membrane filtration. After washing with WT medium, the membrane filters containing the cells were cut in half and placed on dry ice to stop all metabolic activity. The two filter halves were placed on top of each other, with the cell sample between them, and put directly on the saturated paper. The $^{32}\text{P}_i$ standard was also applied to a filter in a similar fashion. After electrophoresis the filters were glued to the paper using bookbinder's glue (courtesy of Barnard Bindery, Medford, Mass.) and the paper was dried in a drying oven. The labeled phosphate was visualized by autoradiography.

RESULTS

Genetic separation of two major P_i transport systems. Previous studies with strains derived from Hfr Cavalli (20) demonstrated that the Pst system, localized at min 83 of the *E. coli* chromosome, was the only major P_i transport system in these strains. Another major P_i transport system, missing from Hfr Cavalli and all of its derivatives, was discovered in several F^- strains. This system, designated Pit, has been localized by conjugation in the *xyl-malA* region of the chromosome (min 76). All cells containing the Pit system are arsenate sensitive in glucose minimal medium, whereas strains dependent on the Pst system are arsenate resistant in this medium (14; R. L. Bennett, Ph.D. thesis, Tufts University, 1973, Medford, Mass.).

To characterize individual P_i transport systems in *E. coli* it was necessary to obtain relatively isogenic strains dependent on only one major P_i transport system. A conjugation experiment (Table 2) was designed using a mutant unable to accumulate P_i as the donor, and a wild-type recipient, containing both major P_i transport systems. The donor cells were unable to utilize P_i and have a requirement for L- α -glycerophosphate as a source of phosphorous. The recipient strain was *glpT* and cannot use L- α -glycerophosphate as a carbon source. The ability to isolate both *pst*⁺ *pit* and *pst* *pit*⁺ recombinants from this conjugation experiment demonstrated that the mutant strain was actually missing the two major P_i transport systems.

In the conjugation experiment selection was for *mtl*⁺ *rpsL* recombinants, and DL- α -glycerophosphate was used as the phosphate source. Cells containing the Pit system are sensitive to

10 mM arsenate when grown on 1 mM P_i in glucose minimal medium, whereas cells dependent on the Pst system continued exponential growth in the same arsenate-containing medium (21). The gene(s) for the Pst system are 96% cotransducible with the *bglR* locus (20), which governs β -glucoside (salicin) utilization (17). For the initial scoring of the cross it was assumed that the recombinants had the same *pst* allele as indicated by their Bgl status.

Four classes of recombinants could be distinguished with the genetic markers used. Class 1 recombinants (*pst* *bglR*⁺ *pit*) would be similar to the donor strain and would be unable to utilize P_i as a phosphate source. Class 2 recombinants (*pst*⁺ *bglR* *pit*) would be able to utilize phosphate since they still possess a functional Pst system, and would be arsenate resistant in phosphate media since they are *pit*. GR5172 was chosen from this class for further study. Class 3 recombinants (*pst* *bglR*⁺ *pit*⁺) would be able to utilize P_i and would be arsenate sensitive. Although most *bglR*⁺ strains would also be *pst*, due to rare recombination between the *bgl* and *pst* genes, some recombinants would not have received the *pst* allele. Class 4 recombinants (*pst*⁺ *bglR* *pit*⁺) would be similar to the parental recipient strain. They would be able to utilize phosphate and would be arsenate sensitive.

Representatives of the four classes of recombinants described above were obtained from the genetic cross. In addition, a fifth class of recombinants was found which was arsenate resistant, able to utilize P_i , and unable to utilize salicin. The expected genotype of this class is *bglR*⁺ *pst*⁺ *pit*⁺, which could result from either recombination between *pst* and *bglR* or a reversion event at *pst*. Recombination between *pst* and *bglR* would imply that three crossovers had occurred during the selection procedure. We have found in other experiments that a mutation causing an inability to utilize P_i is strongly selected against. Therefore it is most likely that this fifth class arose from a reversion event at *pst* in class 1 recombinants. The absence of the Pst system in a representative of class 3 was directly confirmed using P1 transduction. A *bglR*⁺ *pst*⁺ *pit* Hfr Cavalli strain was used as the recipient strain, and the selection was for salicin utilization. The donor strain was a spontaneous salicin-utilizing derivative of GR5178. A class of transductants became unable to utilize P_i , indicating that the donor strain contained a *pst* allele.

Figure 1 shows growth curves obtained in minimal medium with an arsenate-to- P_i ratio of 10 for the representatives of the three classes of recombinants capable of utilizing P_i , the *pst*⁺ *pit*, *pst*⁺ *pit*⁺, and *pst* *pit*⁺ strains. As expected,

TABLE 2. Genetic cross to isolate strains dependent on a single major P_i transport system^a

Donor (UR1)	<i>bglR</i> ⁺	<i>pst</i>	<i>mtl</i> ⁺	<i>pit</i>	<i>rpsL</i> ⁺
	min 82		×		min 74
Recipient (GR2131B)	<i>bglR</i>	<i>pst</i> ⁺	<i>mtl</i>	<i>pit</i> ⁺	<i>rpsL</i>

Class	Phenotype			Expected genotype ^d	No. obtained
	P _i utilization	Arsenate sensitivity ^b	Sal ^c		
1	—	ND	—	<i>bglR</i> ⁺ <i>pst</i> <i>mtl</i> ⁺ <i>pit</i>	7
2	+	R	+	<i>bglR</i> <i>pst</i> ⁺ <i>mtl</i> ⁺ <i>pit</i>	18
3	+	S	—	<i>bglR</i> (<i>pst</i>) <i>mtl</i> ⁺ <i>pit</i> ⁺	30
4	+	S	+	<i>bglR</i> <i>pst</i> ⁺ <i>mtl</i> ⁺ <i>pit</i> ⁺	18
5	+	R	—	<i>bglR</i> ⁺ <i>pst</i> ⁺ <i>mtl</i> ⁺ <i>pit</i>	7

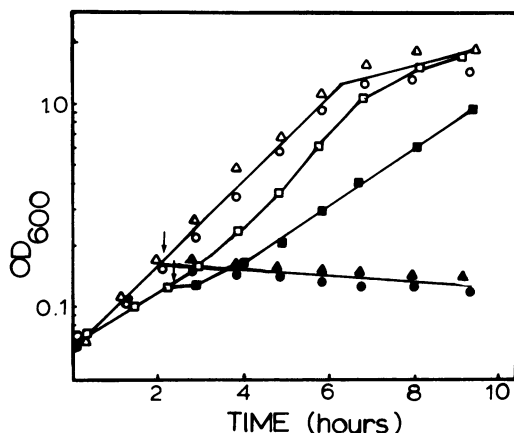
^a Eighty *mtl*⁺ *rpsL* recombinants were scored.^b Arsenate sensitivity was scored on plates with 10 mM arsenate and 1 mM P_i. ND, Not done; R, resistant; S, sensitive.^c The ability to utilize salicin was used to verify the *bgl* status of these strains. Wild-type cells (*bglR*⁺) are unable to utilize salicin, whereas *bglR* mutants can utilize this carbohydrate.^d The marker in parentheses could not be verified in this cross.

FIG. 1. Growth of P_i utilizing recombinants in the presence of arsenate. Stationary-phase cultures in standard minimal salts WT medium supplemented with 0.6% glucose and 1 mM P_i were washed and suspended in fresh medium. At the time indicated by the arrow, the cultures were divided into two portions, one of which received 10 mM arsenate. Growth was followed spectrophotometrically at 600 nm. GR5172, *pst*⁺ *pit*⁻ (□, ■): GR2131, *pst*⁺ *pit*⁺ (○, ●): GR5178, *pst*⁺ *pit*⁺ (△, ▲). Open symbols represent growth without arsenate, whereas closed symbols represent growth in the presence of arsenate.

strains containing a Pit system ceased growth in this arsenate-containing medium. The recombinant which was dependent upon the Pst system for P_i transport continued growth at a slower rate after the addition of arsenate to the medium.

Kinetic parameters for P_i transport. P_i uptake through both major P_i transport systems

requires the presence of a metabolizable energy source, such as glucose (data not shown). Figure 2 shows Lineweaver-Burk plots of the kinetic data used to determine the *K_m* and *V_{max}* values for P_i accumulation through the two major P_i transport systems in the presence of glucose in the medium. Kinetic constants were determined by a nonlinear least-squares analysis minimizing *V* in the Michaelis equation. The error values were determined from the percent relative standard deviation for each curve. The *K_m* for the Pst⁺ Pit⁻ strain was $0.43 \pm 0.02 \mu\text{M P}_i$ and the *V_{max}* was $15.9 \pm 0.3 \text{ nmol of P}_i \text{ (mg [dry weight])}^{-1}(\text{min})^{-1}$, whereas the *K_m* for the Pst⁻ Pit⁺ strain was $38.3 \pm 0.4 \mu\text{M P}_i$ and the *V_{max}* was $55.0 \pm 1.9 \text{ nmol of P}_i \text{ (mg [dry weight])}^{-1}(\text{min})^{-1}$. These data show that the Pst and Pit systems have significantly different kinetic parameters.

The determination of the *K_m* and *V_{max}* values for the wild-type strain, containing both the Pst and Pit transport systems, is shown in Fig. 2C. The apparent *K_m* was $25.1 \pm 1.1 \mu\text{M}$, whereas the apparent *V_{max}* was $43.1 \pm 1.9 \text{ nmol of P}_i \text{ (mg [dry weight])}^{-1}(\text{min})^{-1}$. Since the apparent *V_{max}* for the strain containing both the Pst and Pit systems (Fig. 2C) was lower than the sum of the *V_{max}* values obtained in strains dependent on each of the two systems, a more detailed kinetic analysis was undertaken.

The data was analyzed as being the result of the contributions of each of the major P_i transport systems operating independently. In this case: $V_T = \alpha_{\text{Pst}} V_{\text{Pst}} + \alpha_{\text{Pit}} V_{\text{Pit}}$ (1). In this equation the first term represents the contribution of one system (Pst) to the total observed velocity (*V_T*). α_{Pst} is a constant representing the actual activity of the Pst system, whereas *V_{Pst}* is the theoretical

maximum velocity expected from the Pst system. In a similar manner α_{Pit} and V_{Pit} describe the contribution of the Pit system to the total velocity. α_{Pst} and α_{Pit} can be determined using the Michaelis equation. The results of such an analysis (Fig. 2C) indicate that both systems do not operate independently at full capacity. If both systems were acting independently, then the Pit system is almost fully active, whereas the activity of the Pst system is reduced by an order of magnitude.

Effects of inhibitors on P_i transport. All three classes of phosphate-utilizing recombinants were equally sensitive to the sulfhydryl reagent *N*-ethylmaleimide and the energy uncoupler 2,4-dinitrophenol (Fig. 3). In the pres-

ence of *N*-ethylmaleimide, P_i transport was abolished in all three strains. From 20 to 25% of the $^{32}\text{P}_i$ accumulation was resistant to the uncoupler 2,4-dinitrophenol in all three cases. Sodium cyanide was the only inhibitor that showed a differential effect on the two P_i transport systems (Fig. 4). If NaCN was added at time zero, the strain dependent upon the Pst system for P_i transport was sensitive to this inhibitor, showing only 20% resistant $^{32}\text{P}_i$ uptake in its presence (Fig. 5A). However, if NaCN was added at 2 min after the addition of $^{32}\text{P}_i$, P_i accumulation stopped abruptly. The wild-type strain, like the strain dependent upon the Pst system, showed no cyanide-resistant uptake (Fig. 5B). However, the strain containing only the Pit system for P_i

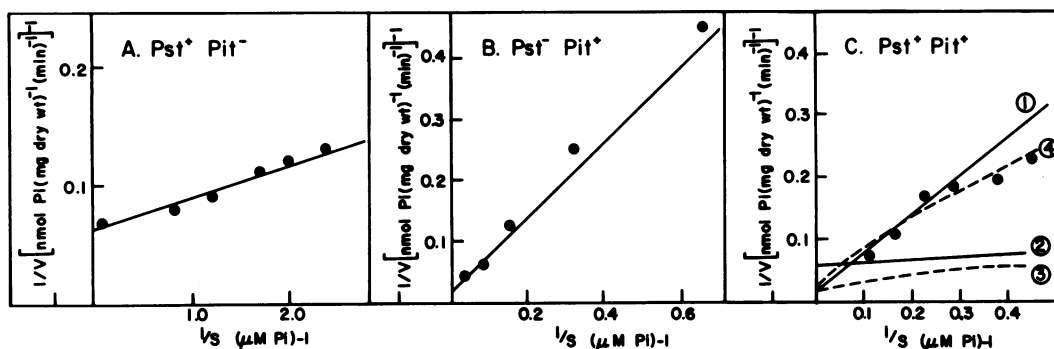


FIG. 2. Kinetics of P_i accumulation in $\text{Pst}^+ \text{Pit}^-$, $\text{Pst}^- \text{Pit}^+$, and $\text{Pst}^+ \text{Pit}^+$ strains. The procedure for P_i transport assays is described in the text. K_m and V_{max} values were determined by a nonlinear least-squares analysis minimizing V in the Michaelis equation. (A) Strain dependent upon the Pst system, GR5172. (B) Strain dependent upon the Pit system, GR5178. (C) Strain containing both P_i transport systems, GR2131B. The following theoretical curves are given: (1) Pit system alone, using the kinetic parameters obtained by the analysis in B. (2) Pst system alone, using the kinetic parameters obtained in the analysis in A. (3) Theoretical curve for $\alpha_{\text{Pst}} = \alpha_{\text{Pit}} = 1.0$, equal contributions of both systems. (4) Theoretical curve for $\alpha_{\text{Pst}} = 0.09$, $\alpha_{\text{Pit}} = 0.9$.

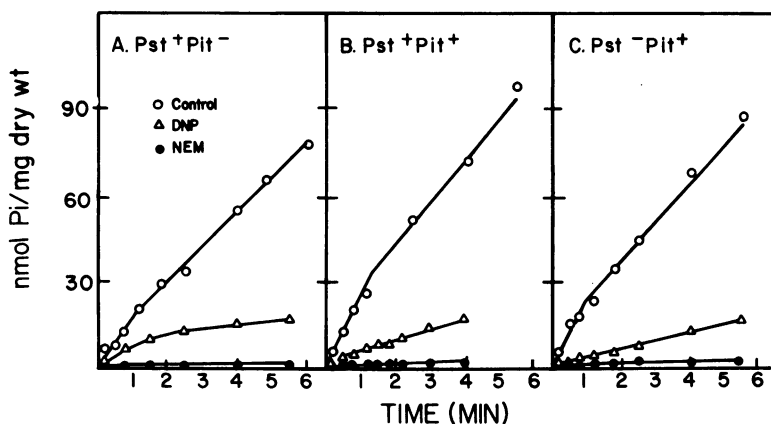


FIG. 3. Sensitivity of P_i uptake to a sulfhydryl reagent and an energy uncoupler. The procedure for P_i transport assays is given in the text. Symbols: \circ , control curves with no additions to the uptake medium; \bullet , 0.5 mM *N*-ethylmaleimide 10 min before the start of the assay; \triangle , 1 mM dinitrophenol was added 3 min before the start of the assay. (A) Strain GR5172 dependent on the Pst system assayed using $30 \mu\text{M } ^{32}\text{P}_i$. (B) Wild-type strain (GR2131B) assayed using $100 \mu\text{M } ^{32}\text{P}_i$. (C) Strain GR5178 dependent on the Pit system assayed using $200 \mu\text{M } ^{32}\text{P}_i$.

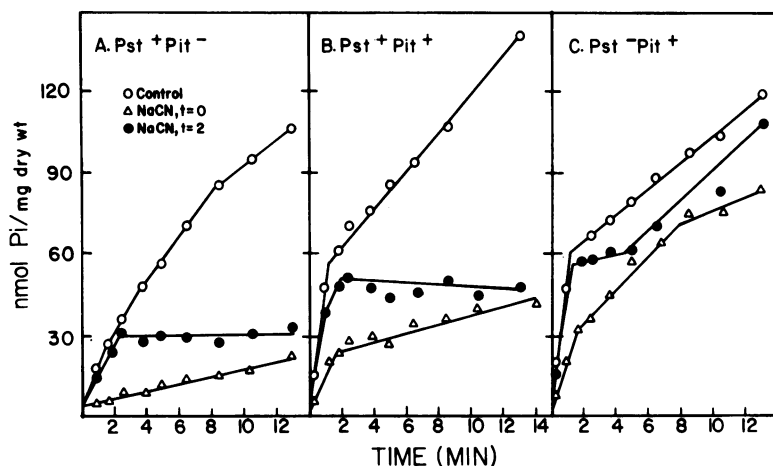


FIG. 4. Sensitivity of P_i uptake to sodium cyanide. For each strain sodium cyanide was added to a final concentration of 1.7 mM at zero time (Δ) or at 2 min (\bullet) after the start of the assay. Assays for all strains contained 300 μ M 32 P_i. \circ , control curve with no additions. (A) Strain dependent on the Pst system (GR5172). (B) Wild-type strain (GR2131B) (both systems present). (C) Strain dependent on the Pit system (GR5178).

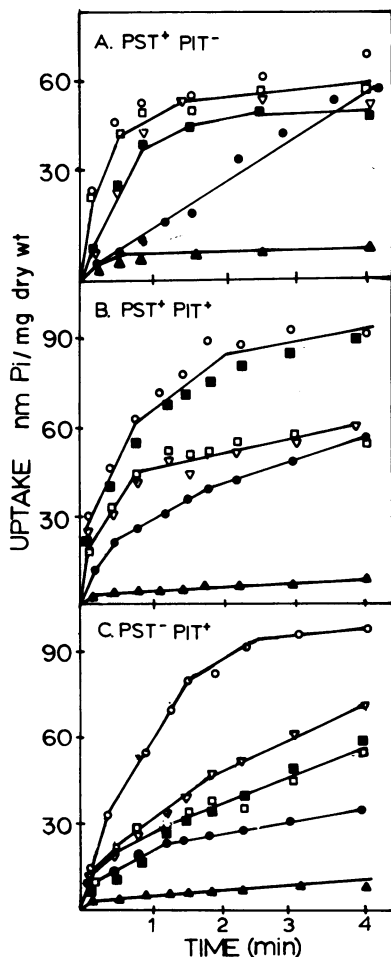


FIG. 5. Effect of various inhibitors on the initial rapid P_i uptake in phosphate-starved cells. Cell sam-

uptake was resistant to NaCN added at zero time, reaching 80% of the control level of 32 P_i accumulation in 13 min (Fig. 4C). If NaCN was added 2 min after the addition of 32 P_i there was a 3-min lag before the accumulation of 32 P_i resumed at a rate similar to the control curve. The cyanide inhibition of the isolated P_i transport systems observed after growth in 1 mM phosphate medium is not consistent with the previously published cyanide inhibition found after P_i starvation (13). To clarify this contradiction, we repeated the inhibitor studies using phosphate-starved cells (Fig. 5). All three strains showed a rapid initial phase of P_i uptake in the first 5 min of the assay. The cyanide inhibition pattern we observed under P_i starvation conditions was the same as that reported in the literature. Thus, the Pst system was basically cyanide resistant, whereas the Pit system appeared to be cyanide sensitive. This pattern of inhibition was also observed with sodium azide. In addition, all of the strains were still sensitive to the energy uncoupler dinitrophenol after phosphate starvation.

Detection of newly transported 32 P_i as P_i. The chemical form of the transported 32 P_i was determined during the assay period (Fig. 6). For

ples were taken at least 2 h after the onset of P_i starvation in cells growing in WT minimal medium with glucose and were assayed using 40 μ M 32 P_i. Symbols: \circ , control (P_i starved); \bullet , control grown in excess P_i medium (1 mM); \blacktriangle , N-ethylmaleimide added to 0.5 mM 3 min before assay; \blacksquare , 1 mM sodium azide added 3 min before assay; \square , 1 mM NaCN added at time zero; ∇ , 1 mM 2,4-dinitrophenol added 3 min before assay. (A) *pst*⁺*pit*⁺ strain GR5172. (B) *pst*⁺*pit*⁺ strain GR2131. (C) *pst*⁺*pit*⁺ strain GR5178.

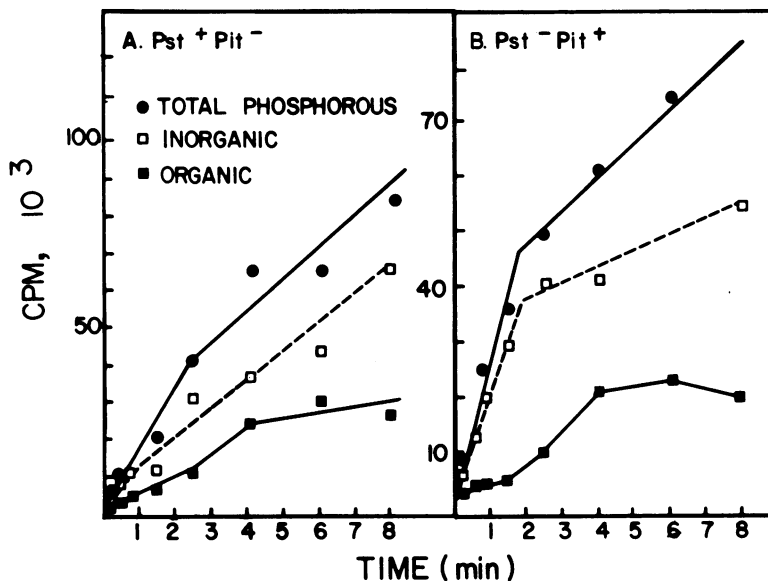


FIG. 6. Measurement of internal P_i in cells accumulating $^{32}P_i$ through different P_i transport systems. The procedure for P_i transport assays given in the text was followed until the cells were washed on membrane filters. The cells were then suspended from the filters into 80°C water. A portion of each sample was dried on planchets and counted directly to give the total $^{32}P_i$ accumulated (●). The remaining sample was centrifuged to remove the cells, and the supernatant was analyzed for P_i as described in the text. A sample of the butanol phase was counted to give total internal P_i extracted (□); a sample of the aqueous phase was counted to give the remaining organic phosphorous (■). All strains were assayed in the presence of 200 μM $^{32}P_i$. (A) Pst^+ strain (GR5172). (B) Pit^+ strain (GR5178).

all strains, at the earliest time point (12 s), the $^{32}P_i$ was found in significant quantities as P_i , indicating that the $^{32}P_i$ entered the cell either as P_i or covalently bound to a rapidly turning over compound. High-voltage electrophoresis at low pH and chromatography at neutral pH (data not shown) of whole cells after exposure to $^{32}P_i$ was also performed. The accumulated $^{32}P_i$ behaved as P_i in the electrophoresis and chromatography experiment. In both systems, after 5 min of accumulation, the label could be seen in nucleosides and high-molecular-weight material that remained at the origin.

Inability of accumulated P_i to exit through the major P_i transport system. After cells had been allowed to accumulate $^{32}P_i$ for 2.5 min, an excess of unlabeled P_i or arsenate was added to the uptake medium. When glucose was the carbon source, neither P_i transport system appeared to function as a P_i exit system (Fig. 7) whether one system alone or both together were involved in the transport of the $^{32}P_i$. Figure 7 also demonstrates that accumulation of $^{32}P_i$ through both the Pst and Pit systems was sensitive to the addition of arsenate.

DISCUSSION

Phosphate transport in P_i -starved cells of *E. coli* through the Pst and Pit systems has been

well characterized by workers in Rosenberg's laboratory (13, 14, 16) who have shown that during P_i starvation the major P_i transport system, Pst , is derepressed by a factor of 5 while the Pit transport system is not derepressed. We were interested in comparing P_i accumulation systems during balanced growth in high-phosphate media or after phosphate starvation (the derepressed state).

R. L. Bennett of this laboratory has described two inducible phosphate ester transport systems which can also transport P_i in *E. coli* (Ph.D. thesis, Tufts University, 1973, Medford, Mass.). One system, induced by growth on glycerol, has been shown to be the *L*- α -glycerophosphate (*glpT*) permease system studied by Lin and co-workers (7). All strains described in this study are genetically missing the *glpT* system. The second system, induced by growth on glucose-6-phosphate, has been shown to be the hexose phosphate permease system (*uhp*) previously studied by Kornberg et al. (6). In the experiments described here cells were never exposed to glucose-6-phosphate, thus the *uhp* system was never induced above the normal low basal level. (In a properly induced strain, both of these systems will catalyze the influx and efflux of both P_i and arsenate.) Isogenic strains dependent upon only one of the major P_i transport

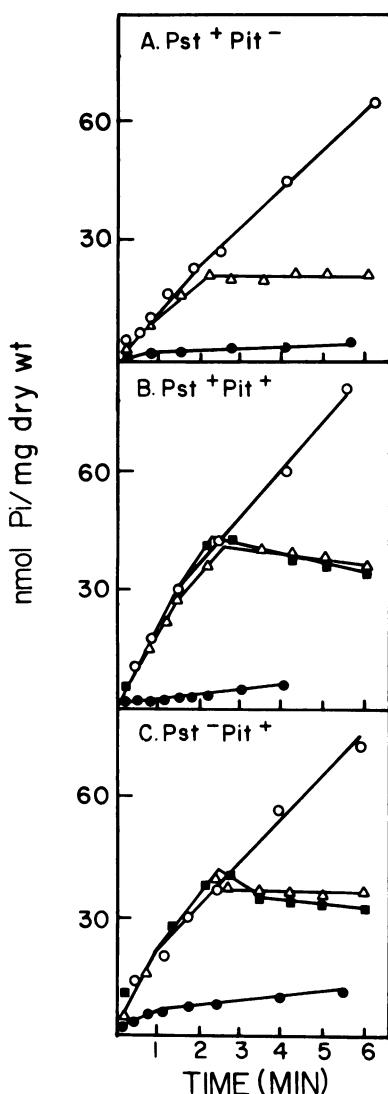


FIG. 7. Lack of exchange of preloaded $^{32}\text{P}_i$ and effect of arsenate on P_i accumulation through the Pst and Pit systems. P_i transport assays were performed as described in the text. Symbols: \circ , control curves with no additions; Δ , nonradioactive 10 mM P_i , or \blacksquare , 10 mM arsenate was added 2.5 min after the start of the assay; \bullet , arsenate to 1 mM was added at zero time. (A) *pst*⁺*pit*⁻ Strain GR5172, assayed using 30 μM $^{32}\text{P}_i$. (B) Wild-type strain, GR2131, assayed using 100 μM $^{32}\text{P}_i$. (C) *pst*⁻*pit*⁺ strain, GR5178, assayed using 200 μM $^{32}\text{P}_i$.

systems were isolated from a conjugation experiment between a donor unable to utilize P_i as sole source of phosphorous and a wild-type Pst⁺ Pit⁺ recipient.

In excess phosphate medium containing glucose as the carbon source the Pst system was a high affinity-low velocity, cyanide-sensitive P_i

transport system. The Pit system had a lower affinity and higher velocity for P_i transport than the Pst system. P_i transport through the Pit system was relatively cyanide resistant. Both systems required the presence of a metabolizable carbon source and were sensitive to 2,4-dinitrophenol, sulfhydryl reagents, and the addition of arsenate to the uptake medium. Neither system functioned as an exit system for newly accumulated $^{32}\text{P}_i$ when cells were grown with glucose as the carbon source. The cyanide sensitivity of the Pst system and resistance of the Pit system observed under these growth conditions were unexpected in light of the results reported in the literature (5, 13, 14).

The energetics of P_i transport has also been studied elsewhere in genetically defined cells. As expected, the Pst system (which involves a binding protein) is not active in membrane vesicles (5). Active transport of P_i is seen in vesicles prepared from Pit-dependent strains and is energized by the respiratory chain. These results imply that the Pst system derives its energy from phosphate bond energy whereas the Pit system is energized by oxidative processes involving a ΔpH (5). On the basis of these observations we would expect that the Pit system should be cyanide sensitive and the Pst system should be resistant. We therefore repeated the cyanide sensitivity experiments with our strains grown under phosphate starvation conditions and found the expected pattern of cyanide inhibition; the Pst system was resistant to cyanide whereas the Pit system was sensitive. More work will have to be done to ascertain if the change in the cyanide response of the Pst and Pit systems in cells growing in excess phosphate media is really indicative of a fundamental change in energization of the two systems.

Our results show that P_i transport in phosphate-starved and high-phosphate-grown cells is always sensitive to the energy uncoupler dinitrophenol, in contrast to the published results in starved cells (13, 14) which report that the Pst system is resistant to the uncoupling agent carbonyl cyanide-*m*-chlorophenyl hydrazine whereas the Pit system is sensitive to this agent.

The ability of cells to exchange internal P_i with extracellular P_i appears to be a function of the carbon source used. Rosenberg et al. (13) have shown that, under P_i starvation conditions, P_i is not exchanged in glucose-grown cells, whereas it is exchanged in cells grown on lactate or succinate. In our experiment using cells grown in excess phosphate medium with glucose as the carbon source, all attempts to isolate the immediate transport product of the Pst and Pit systems have detected only P_i . The observations that newly transported $^{32}\text{P}_i$ cannot be exchanged

through the major P_i transport systems and that the immediate product of transport is P_i would suggest that under these conditions the systems are unidirectional. Differences in P_i exchange characteristics as a function of carbon source have not yet been explained, although it has been suggested that the lack of exchange in glucose-grown cells may be related to the levels of cyclic AMP in these cells.

There appears to be a functional interaction of the Pit and Pst systems in cells grown in excess phosphate medium with glucose as the carbon source. In strains containing both systems, detailed kinetic analysis has shown that either (i) each of the two systems shows fractional activity or (ii) the two systems act as one system with kinetic parameters intermediate to those of each isolated system. Rosenberg's laboratory (13) has reported that, in P_i -starved cells, the effects of the Pst and Pit system are additive. In our experiments using cells grown in excess phosphate medium, the kinetic data is best explained with a 90% contribution of the Pit system and a 10% contribution of the Pst system. In addition, P_i transport in the wild-type strain grown in 1 mM P_i is as cyanide sensitive as in strains containing just the Pst transport system. If the two systems did not interact, one would expect to see the cyanide-resistant activity of the high-velocity Pit system in the wild-type strain containing both P_i transport systems.

Many similarities exist between P_i transport through the Pst and Pit systems in cells grown in high-phosphate media and cells exposed to P_i starvation conditions. It appears that only two major differences can be seen when comparing cells grown in high-phosphate medium with cells exposed to P_i starvation conditions. In excess phosphate medium the Pst and Pit systems appear to interact, whereas this interaction has not been seen in P_i -starved cells. In addition, the response to the energy inhibitor, cyanide, changes with growth conditions. During growth in excess phosphate medium the Pst system is cyanide sensitive, whereas the Pit system is cyanide resistant. After P_i starvation these phenotypes reverse.

In addition to the effect of uncouplers on P_i transport in starved cells discussed above, the response of the Pit system to phosphate starvation was the only other difference that existed between our studies of P_i transport in starved cells and those reported in the literature. In evaluating these results it must be remembered that we used a *pst* mutant isolated as arsenate resistant to inactivate the Pst system, whereas Rosenberg et al. (13, 14, 16) used a *phoT* mutant, isolated as alkaline phosphatase constitutive (see discussion of Willsky and Malamy [21]). We

have reported that these two mutations, although completely inactivating the Pst system for P_i transport, have different rates of constitutive alkaline phosphatase synthesis (20). It is possible that *pst* and *phoT* mutations have different effects on the Pit phosphate transport system. This could explain the differences between our studies and those reported by Rosenberg's laboratory (13).

Sprague et al. (18) isolated mutants that are defective in the function of the Pst and Pit systems using selection procedures designed to obtain mutants impaired in the synthesis and utilization of fatty acids. Their cotransduction experiments have confirmed our report (20) that the *pst* gene(s) map at min 83 of the *E. coli* chromosome. Furthermore, they have also shown by cotransduction that the *pit* gene(s) lies at min 77 of the *E. coli* chromosome. This is in good agreement with the map locus of min 76 obtained in this laboratory by conjugation experiments (this study, R. Bennett, and unpublished observations).

P_i uptake was sensitive to Asi inhibition in all cells not previously exposed to Asi in the growth medium (Fig. 7; unpublished results). However, cells containing only the Pst system will grow in the presence of excess arsenate to P_i in the growth medium (Fig. 1). The accompanying paper deals with characteristics of P_i uptake in a strain dependent on the Pst system grown in high-phosphate minimal medium in the presence of arsenate (21).

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